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Metabolome responses of the sea cucumber *Apostichopus japonicus* to multiple environmental stresses: Heat and hypoxia

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ABSTRACT

Economically important marine organisms face severe environmental challenges, such as high temperature and low dissolved oxygen, from global climate change. Adverse environmental factors impact the survival and growth of economically important marine organisms, thereby negatively influencing the aquaculture industry. However, little is known about the responses of sea cucumbers to combined environmental co-stressors till now. In this study, ultra-performance liquid chromatography (UPLC) was utilized to obtain metabolic profiles of sea cucumbers. Changes in the concentrations of 84, 68, and 417 metabolites related to the responses of sea cucumbers to heat (26 °C), hypoxia (2 mg/L) and the combined stress, respectively, were observed and analyzed. Representative biomarkers were discussed in detail, including deltaline, fusarin C, halichondrin B and rapanone. The concentration of metabolites involved in the regulation of energy metabolism, including amino acid, carbohydrate and lipid metabolism were significantly changed, and the tricarboxylic acid (TCA)-cycle was significantly altered under heat plus hypoxia. We interpreted these changes partly as an adaptation mechanism in response to environmental stress. Based on the decreased accumulation of glutamine, we hypothesized that heat stress is the main factor that interferes with the process of glutamic acid-glutamine metabolism. The present study showed that combined environmental stressors have a more extensive impact on the metabolites of the respiratory tree in sea cucumbers than single stress. These results would facilitate further development of the sea cucumber as an echinoderm model to study mechanisms of response to adverse environments, as well as to help advance knowledge of the adaptation of marine organisms to global climate change.

1. Introduction

Previous research has predicted that global temperatures will rise by at least 2 °C and that the level of dissolved oxygen in the ocean will decline by 4–7% by the end of this century (Hoegh-Guldberg et al., 2007; Matear and Hirst, 2003). Aquatic organisms would be infected disease or even death when the environment factors were far beyond the suitable ranges for them live in, like extremely high temperatures accompanied by low dissolved oxygen. In addition, echinoderms have a relatively narrow tolerance range and cannot survive a multi-day period of anoxia, in contrast to molluscs, anthozoans, ascidians and some other marine species, which can survive without oxygen for several days to a few weeks (Riedel et al., 2012). Thus, massive mortality of sea cucumbers is increasingly common in northern China in summer, and global climate change poses a greater threat to sea cucumbers than to many other aquatic animals. The sea cucumber *Apostichopus japonicus* is an economically important species of echinoderm that has high nutritional and pharmaceutical value and is widely cultured in East Asia (Chang et al., 2004). In 2015, the total output of this species exceeded 205,791 tons in China, with an increase of 2.40% over the 2014 output (Agriculture, 2016). However, sea cucumbers are very sensitive to environmental stress, including high temperature (heat or thermal stress), low dissolved oxygen (hypoxic stress) and low salinity (hyposaline stress). In the summer of 2013, the *A. japonicus* aquaculture industry in China suffered a significant economic loss, and the main causes were identified as heat and hypoxia (Liu et al., 2014; Liu et al., 2016).

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Temperature and dissolved oxygen are two important factors that can influence sea cucumbers at multiple levels, including behavioral, physiological, histomorphological and molecular characteristics. As illustrated by a previous study, the optimal temperature for growth of *A. japonicus* is 10 °C–16 °C (Yuan, 2005). When the ambient temperature gradually increases from 16 °C to 26 °C, the body weight of the sea cucumbers gradually decreases, and the activity levels of antioxidases and heat shock proteins (*Hsp10*, *Hsp60* and *Hsp70*) increase (Ji et al., 2008; Xu et al., 2014). Moreover, as temperatures rise (16 °C–22 °C), food intake, specific growth rate and food conversion efficiency decrease, while the amount of energy required for respiration increases (An et al., 2007). In addition, high water temperatures (above 20 °C) are adverse conditions for the feeding and growth of sea cucumbers, and severe tissue degradation and cellular injury were observed in *A. japonicus* under heat stress (Huo et al., 2018; Xu et al., 2015; Yang et al., 2005).

High temperature also results in decreased oxygen solubility; thus, it is often accompanied by hypoxia in marine ecosystems (Lu et al., 2016). Hypoxia, which is often defined as dissolved oxygen levels below 2 mg/L in aquatic systems, can have lethal or sublethal consequences in sea cucumbers intended for harvest, resulting in degradation of aquaculture resources through enormous mortality of the species (Huang et al., 2012). A survey about the aquaculture area of sea cucumber in the northern of China showed that, the temperature was ranged from 22.66 to 24.98 °C, and the dissolved oxygen was ranged from 1.62 to 8.77 mg/L in the late summer of 2013 (Liu et al., 2014). Thus, it is common for sea cucumber suffering from heat and hypoxia stress in summer. Previous research has illustrated that nonspecific immunity, metabolic enzyme activity, oxidative stress indices and expression of genes and miRNAs would change under hypoxic stress (Huo et al., 2017; Li et al., 2016; Qian, 2011; Zhang et al., 2017; Zheng et al., 2014). The oxygen consumption rate decreased with the decline in DO, and aerobic metabolism was inhibited in sea cucumbers during hypoxia (Li et al., 2016; Qian, 2011). Overall, temperature and dissolved oxygen levels are two major, important environmental factors for aquaculture and may limit the development of the sea cucumber industry. Therefore, understanding the mechanisms by which *A. japonicus* responds to heat and hypoxia is necessary for both characterizing its physiology and preventing risks that may arise during its aquacultural production.

Metabolomics, which examines physiological, developmental, and pathological states through variations in metabolite concentrations, is an emerging approach for assessing the metabolic status of organisms in marine ecosystems under anthropogenic climate change (Jones et al., 2013; Lin et al., 2006). To illustrate how environmental stress influences marine organisms at the metabolic level, several studies have been performed, including studies on mussel (*Mytilus edulis*) exposed to reduced seawater hypoxia, pH, increased temperature, pathogens, pesticides and starvation (Ellis et al., 2014; Tuffnail et al., 2009); Atlantic salmon (*Salmo salar*) under long-term handling stress and elevated temperature (Karakach et al., 2009; Kullgren et al., 2013); Pacific oyster (*Crassostrea gigas*) exposed to elevated pCO_2 (Wei et al., 2015); abalone (*Haliotis diversicolor*) exposed to heat and hypoxia stress (Lu et al., 2016); common carp (*Cyprinus carpio*) subjected to hypoxia (Hallman et al., 2008; Zhou et al., 2000); Atlantic blue crab (*Callinectes sapidus*) under oxidative stress (Schock et al., 2010); and mussel (*Mytilus galloprovincialis*) exposed to environmental pollution (Fasulo et al., 2012). In addition, research has documented the metabolomic response of *A. japonicus* in a variety of contexts, including heat stress (Shao et al., 2015; Xu et al., 2017), estivation (Liu et al., 2016; Yang et al., 2006), regeneration (Sun et al., 2017), skin ulceration syndrome and pathogen challenge (Shao et al., 2013). The above ^{1}H NMR-based metabolomics reference stated that elevated water temperature disturbed enzymes and metabolites involved in energy metabolism (amino acid and carbohydrate) and induced osmotic regulation in intestine of sea cucumber (Shao et al., 2015; Xu et al., 2017). What's more, sea cucumbers might adopt different strategies towards aestivation and short-term acute heat stress (Xu et al., 2017). However, little is known about how high temperature and low dissolved oxygen affect metabolites in the respiratory tree of the sea cucumber and modulate metabolic pathways, and even less is known about the synergistic effects of multiple stressors.

As environmental stressors seldom occur in isolation in nature, the present work applied ultra-performance liquid chromatography (UPLC)-based metabolomics to study metabolic changes in sea cucumbers under the conditions of heat stress, hypoxic stress and the combination of those two stressors. This study identified synergistic effects of multiple stressors on metabolites. The potential biomarkers of heat and hypoxia in sea cucumber were discussed, respectively. Changes in the abundance of metabolites related to the citric acid (TCA) cycle were demonstrated. The trend of metabolites involved in amino acid, carbohydrate, lipid, cofactor and vitamin, and nucleotide metabolic pathways were concluded. The identification of these metabolites in this study provides new insights into the mechanism whereby sea cucumbers respond to two forms of environmental stress (heat and hypoxia).

2. Material and methods

2.1. Animals

Approximately 100 fresh and healthy sea cucumbers (*A. japonicus*) were collected off the coast of Weihai, China; the wet weights of the animals were 90–110 g measured by electronic balance (LT1002B, TIANLIANG, China). After weighing, the sea cucumbers were acclimated in tanks containing aerated, sand-filtered seawater at 16 ± 0.5 °C for one week before use and were fed a commercial diet (Shandong Oriental Ocean Sci-Tech Co. Ltd., Yantai, China) once a day at 8:00 am. Any uneaten feed was removed daily during the acclimation and experimental periods. All animals were then randomly divided into four groups. One group of sea cucumbers was maintained as the normal control (NC) group, with sufficient oxygen in the water (8 mg/L) and a suitable temperature of 16 °C. Another group of sea cucumbers was maintained as the hypoxic treatment group (LO), with low oxygen in the water (2 mg/L) and a suitable temperature of 16 °C. The third group of sea cucumbers was maintained as the heat stress treatment group (HT), with a high temperature of 26 °C and a sufficient dissolved oxygen concentration. The fourth group of sea cucumbers was maintained as the heat and hypoxia treatment group (HL), with a high temperature of 26 °C and a low oxygen concentration (2 mg/L). The acclimation was carried out by gradually increasing the ambient temperature from 16 °C to 26 °C at a speed about 2 °C/h and decreasing the ambient dissolved oxygen level from 8 mg/L to 2 mg/L at a rate about 1 mg/L/h. The moment when the water temperature rose to 26 °C and/or the dissolved oxygen decreased to 2 mg/L was regarded as the initial time. After exposure to stress for 48 h, ten individual sea cucumbers from ten separate tanks per experimental group were dissected promptly, and their respiratory trees were rinsed with sterile water to remove other substances that may affect metabolite measurements before being preserved in liquid nitrogen and stored at −80 °C.

2.2. Temperature and dissolved oxygen regulation

Based on the actual living conditions of sea cucumbers in northern China in summer and the research examining survivability

demonstrated before, we chose 26 °C as the heat stress temperature and 2 mg/L as hypoxic stress dissolved oxygen level in this study (Ji et al., 2008; Xu et al., 2017). The temperature was regulated in the treatment tanks with a 2-kW heating rod as described in previous studies (Xu et al., 2014; Xu et al., 2017). The oxygen level was regulated with a dissolved oxygen control system with real-time monitoring (Huo et al., 2018). Respiratory trees were selected as the target tissue because they are sensitive to changes in environmental factors like dissolved oxygen and temperature (Zhang et al., 2017). UPLC was used to perform metabolomic profiling because of its superior resolution, sensitivity, and separation speed compared to conventional high-field nuclear magnetic resonance (NMR) and gas or liquid chromatography/mass spectrometry (GC–MS, LC–MS) methods (Gonzalez et al., 2012).

2.3. Metabolite extraction

After removal from the −80 °C freezer, the respiratory trees of experimental sea cucumbers were kept at −20 °C for 30 min and then thawed at 4 °C until they had melted. Tissue samples of approximately 25 mg from each sea cucumber were collected in Eppendorf tubes, and then 800 μL of chilled methanol/water (1:1) solution with two steel balls was put into each tube. The samples were homogenized in a TissueLyser at 40 Hz for 5 min. Subsequently, the steel balls were removed, and the Eppendorf tubes were placed at −20 °C for 2 h. The samples were then centrifuged at 25000*g* for 15 min. After centrifugation, the supernatants were collected in Eppendorf tubes for subsequent analysis.

For each analysis, a quality control (QC) sample, prepared by combining equal aliquots of replicates from each sample, was injected regularly throughout the run to monitor the stability of the UPLC-MS platform (Sangster et al., 2006). This QC sample was also used to condition the column at the beginning of each analysis (five injections). The QC sample was injected once after every ten experimental samples and three times at the end to ensure the quality of data assessment.

2.4. UPLC-MS analysis

In this study, a Xevo G2-XS QTOF (Waters, UK) was used to collect the metabolic data. Progenesis QI software (version 2.2, Nonlinear Dynamics, Newcastle, UK) was used to draw the peak information from the raw data, thus recording the characteristics of the metabolites, including *m/z*, retention time and ion area. After removal of low-quality ions (ion deficiency over 50% in QC samples or deficiency over 80% in experimental samples) by the probabilistic quotient normalization (PQN) method, the data were then corrected by the quality-control-based robust LOESS signal correction (QC-RSC) method. After the ions with excessive fluctuations were filtered out (RSD > 30%), the remaining ions were used for further analysis.

2.4.1. Chromatography

All samples were acquired by the LC–MS system through an automated procedure. First, all chromatographic separations were performed using an UPLC system (Waters, UK). An ACQUITY UPLC BEH C18 column (100 mm * 2.1 mm, 1.7 μm, Waters, UK) was used for the reversed phase separation. The column oven was maintained at 50 °C. The flow rate was 0.4 mL/min and the mobile phase consisted of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). The gradient elution conditions were set as follows: 0–2 min, 100% phase A; 2–11 min, 0% to 100% B; 11–13 min, 100% B; 13–15 min, 0% to 100% A. The injection volume for each sample was 10 μL.

2.4.2. Mass spectrometry

A Xevo G2 XS QTOF high-resolution tandem mass spectrometer (Waters, UK) was used to detect metabolites eluted from the column. The apparatus was operated in both positive and negative ion modes. For the positive ion mode, the capillary and sampling cone voltages were set at 3 kV and 40 V, respectively. For the negative ion mode, the capillary and sampling cone voltages were set at 1 kV and 40 V, respectively. The mass spectrometry data were acquired in Centroid MSE mode. The TOF mass range was from 50 to 1200 Da, and the scan time was 0.2 s. For the MS/MS detection, all precursors were fragmented at 20–40 eV, and the scan time was 0.2 s. During the acquisition, the LE signal was acquired every 3 s to calibrate the mass accuracy. Furthermore, to evaluate the stability of the LC–MS during the entire acquisition, a QC sample (pool of all samples) was acquired after every 10 samples.

2.4.3. Multivariate statistics

Multivariate analysis techniques, including unsupervised principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), were used to discriminate among the samples and identify the most important secondary metabolites characterizing their differences (Xiong et al., 2012). PLS-DA is a PLS regression of a set Y of binary variables representing the kinds of a categorical variable on a set X of predictor variables (Perez-Enciso and Tenenhaus, 2003). The quality of the PLS-DA models was described by the cross-validation parameter Q^2 (the predictability of the model) and R^2 (the total explained variation for the X matrix) (Xiong et al., 2012). PCA is an unsupervised multivariate data analysis method that provides a comprehensive view of the metabolic differences between the treatment group and the control group (Xiong et al., 2012). The variable importance in projection (VIP) value was used to represent the importance of variables for the classification (Xiong et al., 2012). Student's *t*-test and fold change analysis were used and the metabolites present at significant levels were identified using Progenesis QI (version 2.2, UK) as the intersection of the following criteria: 1) VIP value ≥ 1; 2) fold change ≥ 1.2000 or fold change ≤ 0.8333 (absolute value of log (treatment/control) ≥ 0.0792); and 3) *p*-value < 0.05. Moreover, the most representative changed metabolites were selected based on the following criteria: a. $p < 0.05$; b. absolute value of log(treatment/control) > 0.25; c. VIP value > 2. Heatmap plots were obtained in R software (version 3.5.1) using the *pheatmap* package (Kolde, 2012). Log_2 transformation of metabolite relative response values was performed prior to heatmap plotting. The area under the receiver operating characteristics (AUC) values, sensitivity (SE) and specificity (SP) of the potential biomarkers on the test set were calculated by R and SPSS19 software (IBM Corp., Armonk, NY, USA) to evaluate the differential performance of metabolites (Hanley and McNeil, 1982).

3. Result

3.1. Differential metabolites identified in the respiratory tree of A. japonicus under environmental stress

In the present study, we conducted a metabolomics study based on UPLC to obtain the different metabolic profiles of the respiratory tree in *A. japonicus* under three different stress conditions (heat, hypoxia and heat plus hypoxia). Supervised PLS-DA was applied to analyze the differences between the treatments and the control, and the scores plot of negative ions is shown in Fig. 1. The parameters R^2 and Q^2, shown in Table S1, indicate the excellent prediction capability of the model. Significantly changed metabolites were selected according to VIP value, fold-change ratio and p-value as described above. The different ion

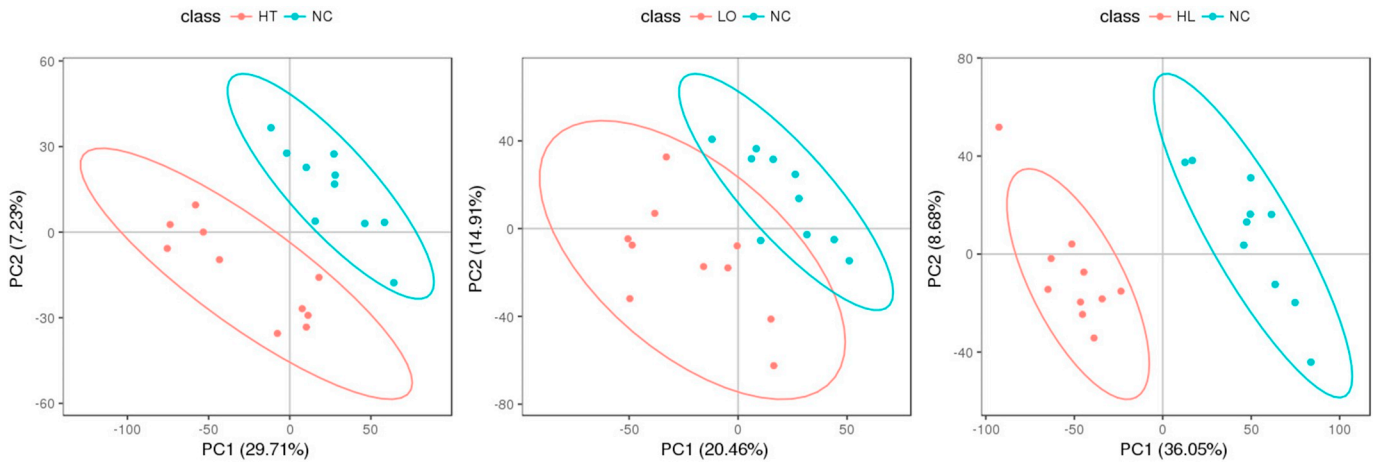

Fig. 1. PLS-DA scores plot of negative ions in all observations. (a. High temperature compared with normal control (HT_NC); b. low dissolved oxygen compared with normal control (LO_NC); c. high temperature plus low dissolved oxygen compared with normal control (HL_NC)).

Table 1
Identified up- and down-regulated negative ions.

Mode	Group	Total ion number	Up (MS)	Down (MS)	Up (MS2)	Down (MS2)
Neg	HT_NC	823	69	129	32	52
Neg	LO_NC	584	76	64	37	31
Neg	HL_NC	2276	129	720	58	359

MS: the number of ions that have been identified after searching the database by the first-level data, which refers to the data displayed on the mass spectrometer after the metabolite is added with ions such as H^+/$NH4^+$/COOH−; MS2: the number of ions after searching through the theoretical secondary fragmentation of the database, which refers to the data of metabolite fragment ions after the primary data has been fragmented in the mass spectrometer.

numbers are shown in Table 1.

In this study, we focused on the metabolites that were identified as differing between the experimental groups (heat, hypoxia and combination groups) and the control group. Only the RT–*m/z* pairs that specifically corresponded to one metabolite were considered variables for that group. As a result, 256, 291 and 534 up-regulated variables and 567, 293 and 1742 down-regulated variables were selected in the comparisons of heat (HT_NC), hypoxia (LO_NC) and the combination vs control (HL_NC), respectively. The shared and specific variables in the different compared sets could be found in Fig. 2. Only 24 up-regulated variables and 14 down-regulated variables were shared by all three sets.

3.2. Metabolic changes in respiratory tree extracts from A. japonicus subjected to different stressors

In total, 84, 68, and 417 significantly different metabolites were identified in sea cucumbers under heat, hypoxia and the combined stress, respectively (Table S2). Among those metabolites, 9, 9 and 78 were identified as the most representative changed metabolites in sea cucumbers in response to heat, hypoxia and the combination, respectively (Table 2). The values of the area under the receiver operating characteristic curve (AUC), sensitivity (SE) and specificity (SP) values in the heat group and the hypoxia group were examined to evaluate the metabolites that change to different degrees in sea cucumbers under various environmental stresses (Table S3). The ten metabolites with the highest AUC values in each group were selected as candidate biomarkers, including five increased and five decreased metabolites.

3.2.1. Metabolic changes in response to heat stress

A total of 84 metabolites were identified as differentially abundant

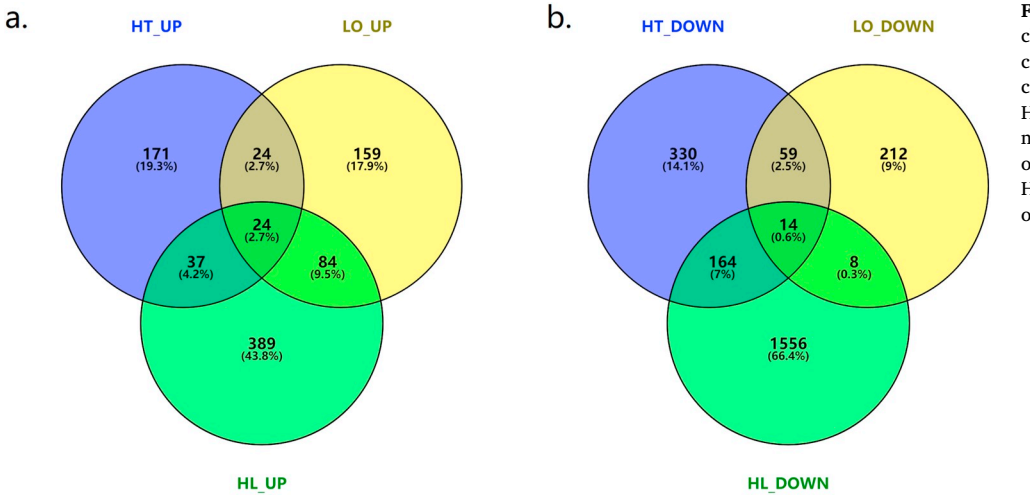

Fig. 2. Numbers of variable metabolites characterizing the respiratory tree samples collected from the HT, LO and HL groups compared to the NC group.
HT_NC: high temperature compared with normal control; LO_NC: low dissolved oxygen compared with normal control; HL_NC: high temperature plus low dissolved oxygen compared with normal control.

Table 2
The representative metabolites showing significantly changed abundance in sea cucumbers under environmental stress.

Groups	Description	Formula	abslog	p-value	VIP	KEGG.ID	regulation
Heat group	Vanillate	C8H8O4	0.3627	0.0311	2.6074	C06672	up
	D-glycero-D-manno-Heptose	C7H14O7	0.2865	0.0252	2.0595	C21042	up
	Gentamicin X2	C19H38N4O10	0.2842	0.0237	2.1678	C17702	up
	Alisol C	C30H46O5	0.2639	0.0093	2.5385	C17461	down
	Valiolone	C7H12O6	0.2734	0.0372	2.0173	C12113	down
	Deltaline	C27H41NO8	0.3317	0.0014	2.2905	C08679	down
	9-cis,11-trans-Octadecadienoate	C18H32O2	0.3428	0.0373	2.6983	C04056	down
	Epothilone A	C26H39NO6S	0.4175	0.0254	2.0877	C12153	down
	Sarin	C4H10FO2P	0.7409	0.0214	3.3612	C11764	down
Hypoxia group	3beta-Chloro-5-androsten -17beta-ol	C19H29ClO	0.2946	0.0033	2.2751	C15396	up
	Myristoleic acid	C14H26O2	0.2912	0.0217	3.1225	C08322	up
	beta-Fabatriose	C18H30O16	0.2790	0.0096	2.3317	C12082	up
	5'-S-Methyl-5'-thioinosine	C11H14N4O4S	0.2768	0.0040	2.3662	C19787	up
	Lupeol acetate	C32H52O2	0.2615	0.0418	2.0092	C08630	down
	Halichondrin B	C60H86O19	0.3906	0.0014	2.8055	C16791	down
	6-Deoxocastasterone	C28H50O4	0.4424	0.0018	2.0632	C15802	down
	Tocopheryl nicotinate	C35H53NO3	0.4733	0.0387	2.8248	C12981	down
Heat and hypoxia group	Molephantin	C19H22O6	0.8670	8.47E-12	2.5635	C09512	up
	Myristoleic acid	C14H26O2	0.6745	0.003316356	2.3461	C08322	up
	Zopolrestat	C19H12F3N3O3S	0.4495	0.032062666	2.2141	C01865	down
	Valclavam	C14H23N3O6	0.5641	0.000740215	2.0128	C06668	down
	WIN I(S)	C21H28N2O3	0.6221	1.26E-05	2.0130	C06497	down
	Tokoronin	C32H52O9	0.6591	0.001725061	2.2273	C08916	down
	Triamiphos	C12H19N6OP	0.6617	8.24E-08	2.0571	C18927	down
	Palmatoside G	C25H32O10	0.6630	1.01E-08	2.0607	C17507	down
	Teleocidin B-1	C28H41N3O2	0.6632	8.43E-05	2.0307	C05150	down
	Antibiotic JI-20B	C20H41N5O9	0.6700	0.000135097	2.0315	C17705	down
	(2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine	C12H23N3O4	0.6837	2.60E-06	2.0308	C20279	down
	3-Cyano-L-alanine	C4H6N2O2	0.6876	7.25E-05	2.0456	C02512	down
	6'''-Deamino-6'''-oxoneomycin C	C23H43N5O14	0.6894	1.32E-05	2.0225	C17589	down
	8-Oxodeoxycoformycin	C11H14N4O4	0.6938	1.67E-05	2.0309	C02957	down
	Farnesylcysteine	C18H31NO2S	0.6988	2.91E-06	2.0422	C19691	down
	3'-Deoxydihydrostreptomycin	C21H41N7O11	0.7142	4.71E-05	2.0800	C03755	down
	Biotin sulfoxide	C10H16N2O4S	0.7151	2.64E-08	2.0774	C20386	down
	Testosterone glucuronide	C25H36O8	0.7244	7.26E-06	2.1457	C11134	down
	Bryotoxin A	C32H42O12	0.7312	4.35E-06	2.0330	C08853	down
	Herbimycin	C30H42N2O9	0.7327	2.30E-05	2.1754	C11225	down
	Mitragynine	C23H30N2O4	0.7328	0.000489526	2.0986	C09226	down
	Valclavam	C14H23N3O6	0.7354	4.07E-06	2.1944	C06668	down
	Glutathione disulfide	C20H32N6O12S2	0.7355	0.00029443	2.2136	C00127	down
	N-Amidino-L-glutamate	C6H11N3O4	0.7362	2.85E-05	2.0613	C03140	down
	17alpha-(N-Acetyl-D-glucosaminyl) -estradiol 3-D-glucuronoside	C32H45NO13	0.7422	5.58E-05	2.1664	C04806	down
	Isotabtoxin	C11H19N3O6	0.7500	9.44E-06	2.1050	C20919	down
	7-Methyluric acid	C6H6N4O3	0.7521	5.46E-08	2.2231	C16355	down
	k-Strophanthoside	C42H64O19	0.7683	1.44E-05	2.1064	C08881	down
	Isotabtoxin	C11H19N3O6	0.7714	3.15E-07	2.1077	C20919	down
	Lyngbyatoxin	C27H39N3O2	0.7780	3.09E-06	2.0507	C15720	down
	Z-Gly-Pro-Leu-Gly-Pro	C28H39N5O8	0.7786	8.81E-05	2.1746	C03183	down
	4,4-Difluoropregn-5-ene-3,20-dione	C21H28F2O2	0.7859	3.20E-07	2.1508	C15152	down
	Tirofiban	C22H36N2O5S	0.7863	0.000206234	2.1805	C07965	down
	Farnesylcysteine	C18H31NO2S	0.7884	6.88E-07	2.0945	C19691	down
	Finaconitine	C33H46N2O10	0.8030	0.000457744	2.3610	C08684	down
	Leukotriene D4	C25H40N2O6S	0.8127	0.014631654	2.5895	C05951	down
	Enicoflavine	C10H13NO4	0.8200	3.17E-05	2.1498	C09946	down
	Dihydrozeatin-O-glucoside	C16H25N5O6	0.8217	9.30E-06	2.2166	C16448	down
	Nicotianamine	C12H21N3O6	0.8288	6.77E-07	2.1548	C05324	down
	3-Hydroxyestra-1,3,5(10)-trien -17-one O-(carboxymethyl)oxime	C20H25NO4	0.8291	1.21E-08	2.2692	C15430	down
	Adiantifoline	C42H50N2O9	0.8403	2.54E-06	2.2353	C09323	down
	Axisothiocyanate 3	C16H25NS	0.8475	3.04E-06	2.2106	C17007	down
	Ouabain	C29H44O12	0.8513	8.02E-06	2.2781	C01443	down
	Rutacridone	C19H17NO3	0.8605	8.75E-09	2.2431	C10738	down
	Sulfoglycolithocholate	C26H42NO7S-	0.8711	5.81E-07	2.3362	C11301	down
	Catharine	C46H54N4O10	0.8815	6.06E-06	2.1047	C09129	down
	Akuammine	C22H26N2O4	0.8929	0.000317013	2.0171	C09027	down
	Megestrol acetate	C24H32O4	0.8974	1.11E-05	2.4649	C08151	down
	1-Octen-3-ol-3-o-beta-D -xylopyranosyl(1- > 6)-beta -D-glucopyranoside	C19H34O10	0.9000	5.56E-08	2.3155	C17614	down

(*continued on next page*)

Table 2 (*continued*)

Groups	Description	Formula	abslog	p-value	VIP	KEGG.ID	regulation
	Aeruginopeptin 228A	C52H68N8O15	0.9017	3.73E-06	2.2085	C15746	down
	Isotabtoxin	C11H19N3O6	0.9082	4.57E-05	2.0297	C20919	down
	D-Lysopine	C9H18N2O4	0.9218	0.011637361	2.0156	C04020	down
	Indicine	C15H25NO5	0.9299	1.24E-05	2.1640	C10326	down
	Acetylgitaloxin	C44H66O16	0.9542	2.02E-05	2.4817	C16749	down
	Axisothiocyanate 3	C16H25NS	0.9555	2.91E-07	2.4817	C17007	down
	Carvedilol	C24H26N2O4	0.9652	0.000256397	2.0291	C06875	down
	Leu-Gly-Pro	C13H23N3O4	0.9676	1.26E-08	2.4757	C01833	down
	Leukotriene D4	C25H40N2O6S	0.9689	5.43E-05	2.3777	C05951	down
	(2R,3R)-3-Methylglutamyl-5 -semialdehyde-N6-lysine	C12H23N3O4	0.9716	1.47E-07	2.3925	C20279	down
	Diltiazem	C22H26N2O4S	0.9868	6.52E-05	2.1944	C06958	down
	Nigakilactone E	C24H34O8	1.0047	5.50E-06	2.2064	C17030	down
	Valclavam	C14H23N3O6	1.0075	2.20E-07	2.5949	C06668	down
	3"-Amino-3"-deoxygentamicin A2	C17H34N4O10	1.0105	8.43E-06	2.2735	C21266	down
	9-Fluoro-16alpha-hydroxyandrost -4-ene-3,11,17-trione	C19H23FO4	1.0307	2.66E-06	2.4710	C15105	down
	2,4-Bis(acetamido)-2,4,6-trideoxy -beta-L-altropyranose	C10H18N2O5	1.0427	0.000243747	2.0479	C19972	down
	Glycopeptide	C36H64N8O17	1.0598	0.000119659	2.1683	C00528	down
	Dapdiamide A	C12H20N4O5	1.1079	0.000305157	2.7002	C20962	down
	Pristinamycin IB	C44H52N8O10	1.1092	7.42E-05	2.5470	C11616	down
	Dolichyl diphosphate	C20H38O7P2. [C5H8]n	1.1523	5.87E-06	2.5800	C00621	down
	Leu-Gly-Pro	C13H23N3O4	1.1552	4.53E-06	2.9086	C01833	down
	L-Alanyl-gamma-D-glutamyl-L-lysine	C14H26N4O6	1.1671	1.69E-05	2.6194	C21160	down
	Pelargonidin 3-O-rutinoside 5 -O-beta-D-glucoside	C33H41O19+	1.2085	2.99E-05	2.7435	C12645	down
	Cyenopyrafen	C24H31N3O2	1.2391	0.000679241	2.8350	C18545	down
	Coleonol	C22H34O7	1.2523	0.00017474	3.0144	C09076	down
	Penitrem E	C37H45NO6	1.2746	3.83E-05	3.1466	C20597	down
	Taurohyocholate	C26H45NO7S	1.2812	0.001004138	3.0367	C15516	down
	Leu-Gly-Pro	C13H23N3O4	1.2880	1.55E-05	2.9319	C01833	down
	Pravastatin	C23H36O7	1.5404	1.58E-05	3.4134	C01844	down

m/z: mass-to-charge ratio; abslog: absolute value of log(treatment/control); VIP: variable importance in projection.

under heat stress. Among the nine most representative changed metabolites in sea cucumbers under heat stress, three metabolites were increased, namely, vanillate, D-glycero-D-manno-heptose and gentamicin X2, and six metabolites were decreased, namely, alisol C, valiolone, deltaline, 9-*cis*,11-*trans*-octadecadienoate, epothilone A and sarin. These metabolites are involved in various pathways, such as microbial metabolism in diverse environments; Type I polyketide structures; biosynthesis of antibiotics; aminobenzoate degradation; and linoleic acid metabolism. The concentrations of ten candidate biomarkers in sea cucumbers under heat stress were analyzed by hierarchical clustering and are shown in Fig. 3a. Among the candidate biomarkers, deltaline, fusarin C, cyclopiazonic acid, (*R*)-5,6-dihydrothymine and oxaloglutarate were significantly increased; acroptilin, prorocentrolide B, ophiobolin A, 11-dehydrocorticosterone and 7-epiloganic acid were significantly decreased.

3.2.2. Metabolic changes in response to hypoxic stress

A total of 68 metabolites were identified as differentially abundant under hypoxic stress. Ten representative changed metabolites, including six increased metabolites (volicitin, 3-beta-chloro-5-androsten-17-beta-ol, myristoleic acid, beta-fabatriose and 5′-*S*-methyl-5′-thioinosine) and four decreased metabolites (lupeol acetate, halichondrin B, 6-deoxocastasterone and tocopheryl nicotinate), are involved mainly in metabolic pathways, such as cysteine and methionine metabolism, alpha-linolenic acid metabolism, vitamin digestion and absorption, brassinosteroid biosynthesis and biosynthesis of secondary metabolites. The concentrations of ten candidate biomarkers in sea cucumbers under hypoxic stress were analyzed by hierarchical clustering and are shown in Fig. 3b. Among the candidates, halichondrin B, absinthin, 8′-apo-beta-carotenal, crotonobetainyl-CoA and 6-deoxocastasterone were significantly increased; *N*-glycoloyl-neuraminate, rapanone, beta-fabatriose, (25*S*)-3-oxocholest-4-en-26-oyl-CoA and volicitin were significantly decreased.

3.2.3. Metabolic changes in response to combined stress of heat and hypoxia

A total of 417 differentially expressed metabolites were identified under heat plus hypoxia. Among the 78 representative changed metabolites of sea cucumbers under heat plus hypoxia, two were increased, including molephantin and myristoleic acid; 76 were decreased, including valclavam, (2*R*,3*R*)-3-methylglutamyl-5-semialdehyde-N6-lysine, farnesylcysteine, isotabtoxin, leukotriene D4, axisothiocyanate-3 and Leu-Gly-Pro. These metabolites were involved in a variety of pathways based on KEGG database, such as biosynthesis of antibiotics; biosynthesis of secondary metabolites; bile secretion; and terpenoid backbone biosynthesis.

Moreover, most potential biomarkers were not significantly changed under heat plus hypoxia, except that Cyclopiazonic acid were significantly down-regulated; Rapanone and alpha-L-Rhamnopyranosyl-(1- > 2)-beta-D-galactopyranosyl-(1- > 2)-beta-D-glucuronopyranoside were significantly up-regulated. Furthermore, the identified temperature-biomarkers were not significantly accumulated in hypoxia group and oxygen-biomarkers were not significantly accumulated in heat group. These results showed specificity of identified potential biomarkers to the type of stress.

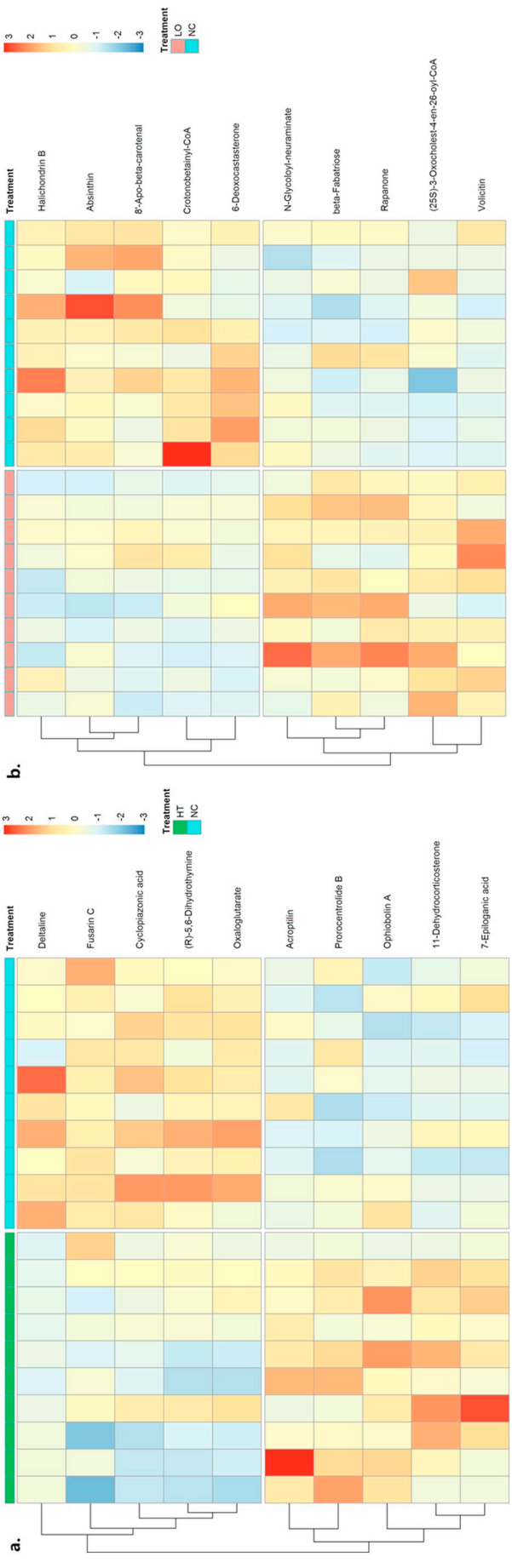

Fig. 3. Heatmap of candidate biomarkers of heat and hypoxia in sea cucumbers.
Red indicates higher levels of metabolites and blue indicates lower levels of metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

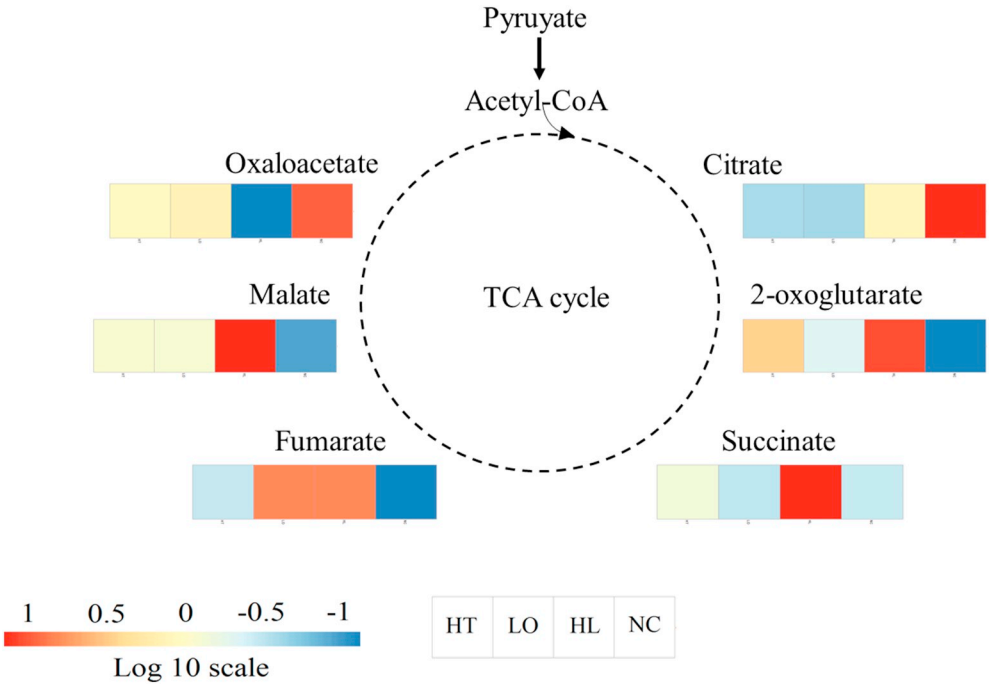

Fig. 4. Relative abundance of characteristic metabolites in the TCA cycle of sea cucumbers under different treatments. The squares from left to right represent four treatments (HT: heat treatment; LO: hypoxic treatment; HL: heat plus hypoxia treatment; NC: normal conditions). The colors represent the pairwise correlation coefficients ranging from 1 (red) to −1 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Expression of key metabolites involved in the TCA cycle

As the key regulators of carbon and nitrogen metabolism, metabolites involved in the TCA cycle are of great importance because of their interactions with multiple metabolic networks. The relative concentrations of characteristic metabolites in the TCA cycle, including oxaloacetate, malate, fumarate, citrate, 2-oxoglutarate and succinate, in sea cucumbers subjected to different treatments are shown in Fig. 4. Compared with the control group, the levels of oxaloacetate and citrate were decreased under heat and/or hypoxia treatment. However, the levels of malate, 2-oxoglutarate, and fumarate were increased under those treatments. The level of succinate was higher in the combined stress group than in the other groups. These results showed that metabolites in the TCA cycle were impacted by environmental stress.

Table 3
The numbers of significantly changed metabolites involved in common pathways under three environmental stress conditions.

Pathway	Category	HT_NC	LO_NC	HL_NC
Metabolic pathways	Global metabolism	47	28	171
ABC transporters	Membrane transport	2	4	21
Cysteine and methionine metabolism	Amino acid metabolism	1	1	11
Tryptophan metabolism	Amino acid metabolism	2	1	13
Histidine metabolism	Amino acid metabolism	4	2	11
Starch and sucrose metabolism	Carbohydrate metabolism	6	4	6
Arachidonic acid metabolism	Lipid metabolism	1	16	15
Steroid hormone biosynthesis	Lipid metabolism	6	1	7
Purine metabolism	Nucleotide metabolism	8	1	12
Pyrimidine metabolism	Nucleotide metabolism	6	1	11
Porphyrin and chlorophyll metabolism	Metabolism of cofactors and vitamins	3	3	16
Monobactam biosynthesis	Biosynthesis of other secondary metabolites	4	2	13
Bile secretion	Digestive system	6	6	14
Serotonergic synapse	Nervous system	2	5	12
Neuroactive ligand-receptor interaction	Signaling molecules and interaction	1	7	10

3.4. Key differentially activated pathways in sea cucumbers under environmental stress

The metabolites identified as significantly changed were involved in various pathways, and the top 15 pathways in the negative ion model shared by all the three environmental stress conditions are listed in Table 3 and include metabolic pathways, ABC transporters and porphyrin and chlorophyll metabolism. Across various pathways in the sea cucumber, more metabolites were altered under heat plus hypoxia than under either form of stress alone, indicating that the interaction between these two stressors has an additive effect on a metabolic level and that the process through which the sea cucumber responds to these combined stressors is more complicated than the response to either component.

3.4.1. Amino acid metabolism

Significantly changed metabolites involved in amino acid metabolism were analyzed by hierarchical clustering and are shown in Fig. 5. In sea cucumbers under hypoxic treatment, 5′-*S*-methyl-5′-thioinosine (C19787) and *N*-formimino-L-glutamate (C00439) were significantly increased, indicating a high level of amino acid metabolism. Moreover, sea cucumbers under heat stress showed significantly increased 2-hydroxy-6-oxonona-2,4-diene-1,9-dioate (C04479) and decreased L-glutamine (C00064), which are involved in amino acid metabolism. In sea cucumbers under heat plus hypoxia, other than the significantly increased gamma-L-glutamyl-*S*-(hercyn-2-yl)-L-cysteine *S*-oxide (C20995), most metabolites involved in amino acid metabolism were significantly decreased, such as L-aspartic 4-semialdehyde (C00441), L-glutamine (C00064) and sulfate (C00059).

3.4.2. Carbohydrate metabolism

The significantly changed metabolites involved in carbohydrate

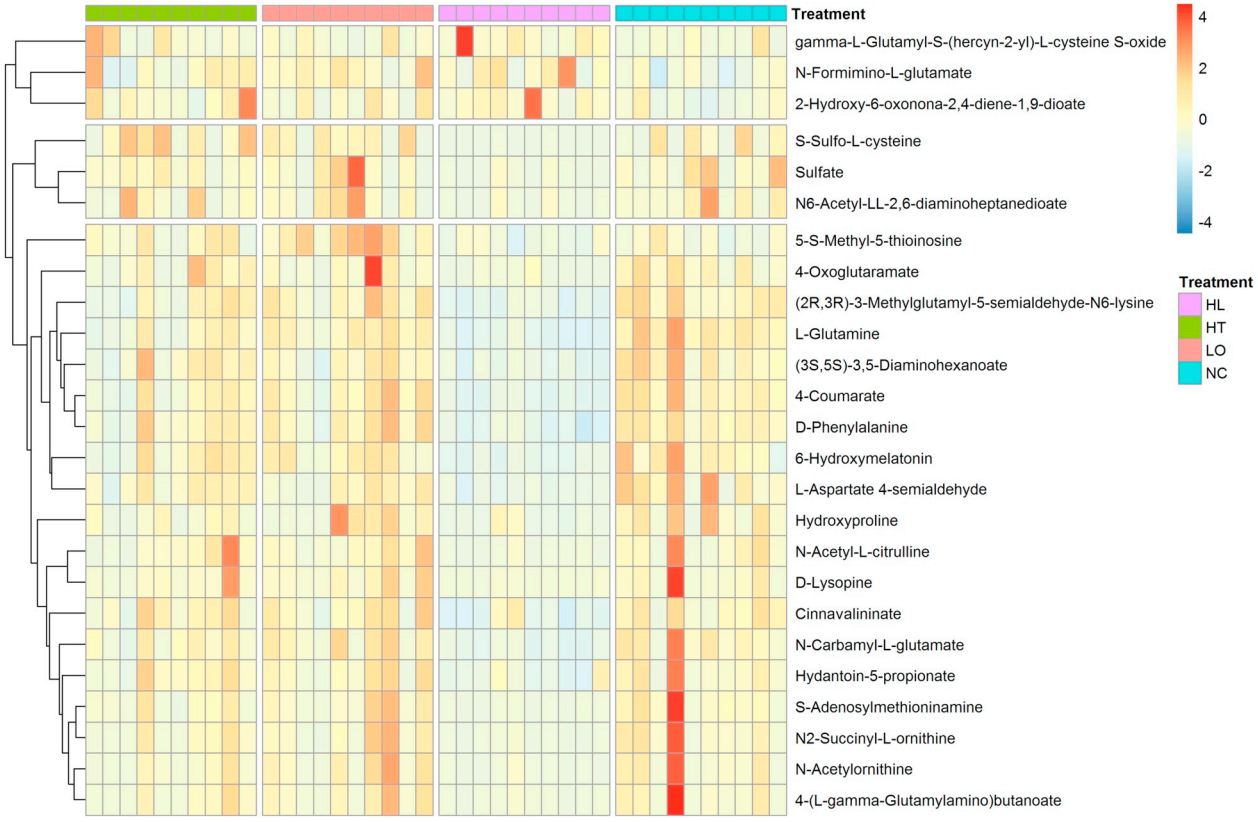

Fig. 5. Heatmap plot of changed metabolites involved in amino acid metabolism in sea cucumbers under environmental stress.

metabolism were analyzed by hierarchical clustering and are shown in Fig. 6. Most of the metabolites decreased in abundance under environmental stress, especially under heat plus hypoxia, and only a few metabolites increased in abundance under environmental stress. Regarding the carbohydrate metabolism pathway, *N*-glycoloyl-neuraminate (C03410) was significantly increased in sea cucumbers under

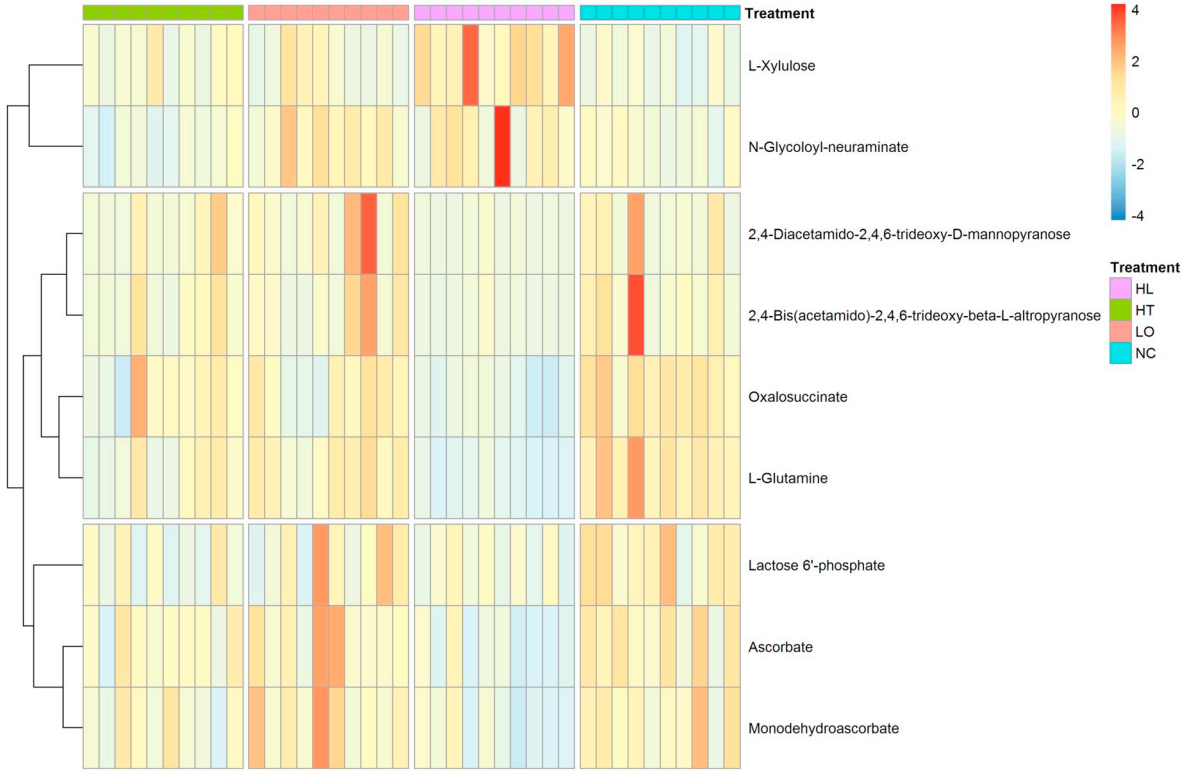

Fig. 6. Heatmap plot of changed metabolites involved in carbohydrate metabolism in sea cucumbers under environmental stress.

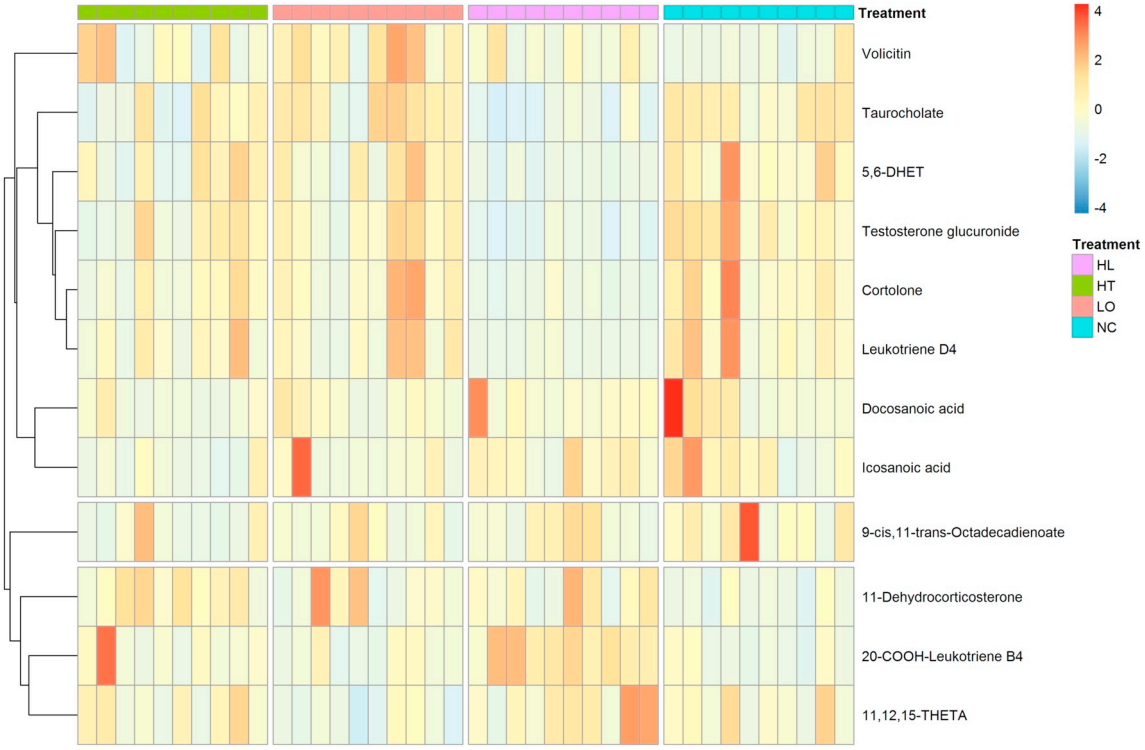

Fig. 7. Heatmap plot of changed metabolites involved in lipid metabolism in sea cucumbers under environmental stress.

hypoxic stress, and lactose 6′-phosphate (C05396) was significantly decreased in sea cucumbers under heat stress. Under the combined stress, other than the increased level of L-xylulose (C00312), most significantly changed metabolites were decreased, including 2,4-bis(acetamido)-2,4,6-trideoxy-beta-L-altropyranose (C19972) and 2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose (C20424).

3.4.3. Lipid metabolism

Significantly changed metabolites involved in lipid metabolism were analyzed by hierarchical clustering and are shown in Fig. 7. Under hypoxic stress, volicitin (C16345) was significantly increased, while 11,12,15-THETA (C14782) was significantly decreased. Under heat stress, 11-dehydrocorticosterone (C05490) was significantly increased, but icosanoic acid (C06425), 9-*cis*,11-*trans*-octadecadienoate (C04056) and docosanoic acid (C08281) were significantly decreased. Moreover, under heat plus hypoxia, 20-COOH-leukotriene B4 (C05950) was significantly increased. Other significantly changed metabolites were decreased, including icosanoic acid (C06425), leukotriene D4 (C05951) and taurocholate (C05122).

3.4.4. Metabolism of cofactors, vitamins, nucleotides and biosynthesis of other secondary metabolites

Significantly changed metabolites involved in metabolism of cofactors and vitamins, nucleotide metabolism and biosynthesis of other secondary metabolites were analyzed by hierarchical clustering and are shown in Figs. S1a, b and S2. Among metabolites involved in the metabolism of cofactors and vitamins, 5,6-dihydrouracil (C00429) was significantly decreased under heat stress. Pantothenate (C00864) was significantly increased under heat stress and the combined stress of heat and hypoxia. Under the combined stress, most of the changed metabolites involved in the metabolism of cofactors and vitamins were observed to decrease, including maleamate (C01596), cobinamide (C05774), 4-coumarate (C00811) and biotin sulfoxide (C20386). Among the metabolites involved in nucleotide metabolism, most of them were significantly decreased. For example, GMP (C00144), 3′,5′-cyclic GMP (C00942), and 2′,3′-cyclic UMP (C02355) were significantly decreased under heat stress. Under the combined stress, UMP (C00105), cytidine (C00475) and deoxyinosine (C05512) were significantly decreased. However, UDP (C00015), which is involved in pyrimidine metabolism, was significantly increased under the combined stress. In the class of biosynthesis of other secondary metabolites, metabolites were significantly decreased under hypoxic stress (6‴-deamino-6‴-oxoneomycin C (C17589)) and the combined stress (3″-amino-3″-deoxygentamicin A2 (C21266), 6′-oxolividamine (C21256) and 7-methyluric acid (C16355)) but increased under heat stress (gentamicin X2 (C17702) and isotabtoxin (C20919)).

4. Discussion

Water temperature and dissolved oxygen are two of the most important limiting factors for *A. japonicus* aquaculture. Extensive mortality of sea cucumbers has occurred in the coastal waters and ponds of China in recent years due to the unsuitable environment. Therefore, there is a pressing need to clarify the responses of sea cucumbers to changed environments. Metabolomics is a valuable tool for identifying and characterizing changes in metabolites, thus revealing physiological alterations in aquatic animals under environmental stress. The present study employed UPLC to characterize the metabolomic responses of the respiratory tree of *A. japonicus* to four different treatments (heat treatment, hypoxia treatment, heat plus hypoxia treatment, and normal conditions). We identified ten potential biomarkers for heat stress and ten for hypoxic stress in sea cucumbers. What's more, the metabolomic trends in response to different forms of environmental stress were determined. As the results showed that, heat stress leads to a decreased

carbohydrate and metabolism and changes in metabolites involved in amino acid, lipid, cofactors and vitamins metabolism. Moreover, when exposed to hypoxia, metabolites involved in amino acid, carbohydrate metabolism and the levels of fatty acids were significantly increased in sea cucumbers. Furthermore, we found that the metabolites responding to the combined stresses were far more numerous, and most metabolites were significantly decreased. TCA cycle and glutamic acid-glutamine metabolic process were also significantly impacted. These results would help to understand the responsive mechanisms of aquatic animals to adverse environments.

4.1. Metabolic changes in sea cucumbers under heat stress

Heat stress, which occurs when the ambient temperature exceeds the range suitable for the metabolism of an organism, can cause metabolic disorders. In this study, some important metabolite biomarkers were discovered in sea cucumbers exposed to high-temperature environment. Deltaline, a type of diterpenoid alkaloid found in *Delphinium Occidentale* (Couch, 1936), was significantly decreased in sea cucumbers under heat stress. A previous study suggested that deltaline may modulate the concentration of methyllycaconitine in serum and, as a result, increase toxicity in cattle (Green et al., 2011). Fusarin C is a mycotoxin and can be produced by *Fusarium* species. It was demonstrated to have stimulative effects on the growth of the breast cancer cell line MF-7 (Sondergaard et al., 2011). In the present study, this metabolite was significantly decreased under heat stress and was identified as a potential biomarker. Acroptilin is a sesquiterpene lactone from *Acroptilon repens* (Evstratova et al., 1967), and Prorocentrolide B can produce a rapid toxic response (Hu et al., 1996). These two metabolites were significantly increased in sea cucumbers under heat stress. However, the above metabolites are not commonly discussed in the literature on metabolic profiles, and the exact functions of these metabolites involved in the defense of *A. japonicus* against heat stress require thorough investigation.

In addition, under heat stress, metabolites involved in amino acid metabolism, lipid metabolism and metabolism of cofactors and vitamins were significantly changed in sea cucumbers. The situation is similar to the findings of a previous study, which reported decreases in several amino acids and a shift in lipid metabolism in Atlantic salmon (*Salmo salar*) at high temperatures (Kullgren et al., 2013). Such changes in amino acid metabolism might be partially explained by the increased ammonia excretion rate in marine organisms at high temperatures, as was reported in *A. japonicus* and *Oplegnathus fasciatus* (Yan et al., 2008; Yang et al., 2006). Conversely, metabolites involved in carbohydrate and nucleotide metabolism, such as lactose 6′-phosphate, GMP and cGMP, were significantly decreased in sea cucumbers under heat stress. Previous studies reported that *A. japonicus* became less active and stopped feeding once the ambient water temperature exceeded 18 °C (Liu et al., 1996; Sui and Liao, 1988; Yang et al., 2005). Thus, together with inactive behavior and decreased carbohydrate and nucleotide metabolism, the sea cucumber might adopt these adaptive strategies to conserve energy in adverse environments.

4.2. Metabolic changes in sea cucumbers under hypoxic stress

As reported previously, global warming may result in reduced oxygen content in the world's oceans (Keeling and Garcia, 2002). Hypoxia is a common disadvantageous environmental feature in aquatic systems worldwide (Lardon et al., 2013). In the present study, some important metabolite markers and key differentially activated pathways were identified in sea cucumbers subjected to low dissolved oxygen levels. Among the identified potential biomarkers of sea cucumber under hypoxic stress, halichondrin B was significantly increased, and rapanone and volicitin were significantly decreased. Halichondrin B is a natural large polyether macrolide isolated from the marine sponge *Halichondria okadai* (Uemura et al., 1985). Because its mechanism of action consists of interacting with tubulin and causing destabilization of the tubulin macromolecule, halichondrin B has been investigated as a potential antitumor agent (Bai et al., 1991; Newman, 2016). Rapanone is a hydroxyl benzoquinone with a special chelating structure. Rapanone was reported to prevent mitochondrial damage by iron or free radicals and is involved in the redox mechanism of the Fenton and Haber-Weiss reactions as an oxidizing agent (de la Vega-Hernandez et al., 2017). Its biological functions have been studied in terms of anti-inflammatory (Ospina et al., 2001), anthelmintic (da Costa et al., 2014) and cytotoxic activity (Cordero et al., 2004). Volicitin is an elicitor isolated from beet armyworm oral secretion and can active genes for the synthesis of terpenoids (Alborn et al., 1997; Shen et al., 2000). A previous study has demonstrated that volicitin-related compounds evoke some defensive responses in plants (Sawada et al., 2006). Such a defensive role for these potential markers has not been reported among marine invertebrates. Succinate, a key metabolite involved in the TCA cycle, has marked stabilizing effects on protein structure (Bowlus and Somero, 1979), and it was identified as upregulated under hypoxia in some shellfish, including *M. edulis* and *M. galloprovincialis* (Hines et al., 2007; Tuffnail et al., 2009). However, succinate was not significantly accumulated in sea cucumbers under hypoxia. A previous study on anaerobic glycolysis in the sea cucumber *Sclerodactyla briareus* did not show succinate or volatile acids to be significant end products (Ellington and Hammen, 1977). We suspect that the situation is similar in *A. japonicus*.

In addition, under hypoxic treatment, metabolites involved in amino acid and carbohydrate metabolism were significantly increased in sea cucumbers. In a previous study on *Thaumatotibia leucotreta*, amino acid metabolism was reported to be significantly altered at the metabolic level by oxygen availability (Boardman et al., 2016). Furthermore, aquatic invertebrates use high concentrations of amino acids to balance intracellular osmolarity with that of the marine environment (Viant et al., 2003). Lipids are a significant energy source for animals (Ru et al., 2017). In the present study, the levels of fatty acids, including unsaturated fatty acids (myristoleid acid), were increased under hypoxic stress. This occurrence may be a result of altered lipid metabolism (decreased lipid oxidation or increased lipid biosynthesis) (Brose et al., 2014). The upregulation of these energy-related metabolites indicates increased energy turnover in sea cucumbers enduring adversely hypoxic environments.

4.3. Metabolic changes in sea cucumbers under heat plus hypoxia

In the natural environment, water temperature and dissolved oxygen are two common factors that directly influence the physiology and survival of aquatic animals. Moreover, heat stress usually occurs simultaneously with hypoxic stress. As has been reported, the availability of ambient oxygen is closely related to the heat sensitivity of animal organisms (Portner et al., 2005). An interactive effect of temperature and dissolved oxygen on the proteomic profile has been reported in some organisms, such as sea cucumber and broccoli sprouts, in terms of protein expression (Guo et al., 2017) [Huo da et.al unpublished paper]. In the present study, the additive effects of these two factors were observed at the metabolic level.

In the present study, the metabolites responding to the combined effects of heat and hypoxia were far more numerous than those responding to either single stressor. This situation indicates the comprehensive nature of the metabolic adjustments that sea cucumbers

undergo when exposed to global climate change. Most metabolites involved in amino acid, carbohydrate and lipid metabolism were significantly decreased under heat plus hypoxia, although L-xylulose (a metabolite related to carbohydrate metabolism) and 20-COOH-leukotriene B4 (a metabolite related to lipid metabolism) were upregulated. L-Xylulose is a reducing sugar that can decrease blood glucose by inhibiting α-glucosidase (Muniruzzaman et al., 1996) [https://pubchem.ncbi.nlm.nih.gov/compound/22253], and is involved in certain metabolic pathways (Meng et al., 2016). This sugar belongs to a class of compounds known as rare sugars, which are monosaccharides and monosaccharide derivatives that rarely exist in nature (Granstrom et al., 2005). In addition, L-xylulose can be used for medical and clinical diagnosis of conditions, including acute or chronic hepatitis and liver cirrhosis (Oka et al., 1976). Furthermore, metabolites involved in the biosynthesis of other secondary metabolites were significantly decreased under heat plus hypoxia; hypoxic stress produced similar results, but heat stress produced the opposite effect. Oxalosuccinate is an alpha-keto compound, produced during the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (Akram, 2014) [HMDB, http://www.hmdb.ca/metabolites/HMDB0003974]. As an unstable 6-carbon intermediate, oxalosuccinate was significantly decreased in sea cucumbers under heat plus hypoxia, indicating significant effects on the TCA cycle. The TCA cycle is affected by a variety of stress-inducing stimuli such as heat, ethanol, and high concentrations of salt via changing related enzymes, genes and intermediates in rice, soybean and *Staphylococcus aureus* (Glaubitz et al., 2015; Sicher, 2013; Tomlins et al., 1971; Vuong et al., 2005). Furthermore, recent reports have identified that lipids, proteins, and nucleic acids precursors are derived from the TCA cycle. Thus, the decrease of most metabolites involved in energy-related metabolism and impressed TCA cycle could be partially explained (Yang et al., 2014).

The functions of glutamine include acid-base balance; immunity; and synthesis of nucleotides, nucleic acids and proteins (Newsholme et al., 2003). Glutamine can suppress the generation of free radicals, reduce the inflammatory response and facilitate the production of heat shock proteins (Wischmeyer et al., 2003). Moreover, it can compensate for glucose to maintain TCA cycle function in absence of pyruvate import (Yang et al., 2014). A previous study indicated that hypoxia shifts glutamine metabolism from oxidation to reductive carboxylation, and it has been proposed to be a mechanism for generating citrate for lipid synthesis when glucose entry into the TCA cycle is reduced (Le et al., 2012; Sun and Denko, 2014). In previous studies, hypoxia alters glutamine metabolism in the immature rat brain and neuronal cell from fetal mice (Krajnc et al., 1996; Sher and Hu, 1990). Moreover, at high temperatures, the level of glutamine was significantly decreased in Atlantic salmon (*Salmo salar*) (Kullgren et al., 2013) and in the intestine and muscles of sea cucumbers (Xu et al., 2017). Indeed, glutamine was thought to play a role in enhancing the heat shock response and even avoiding the triggering of autophagy via its entrance into the hexosamine biosynthetic pathway (HBP) (Leite et al., 2016). In the present study, glutamine was significantly decreased under heat and heat plus hypoxia, indicating that heat stress is the main factor that interferes with the glutamic acid - glutamine metabolic process.

5. Conclusion

In the present study, UPLC-based metabolomics was applied to identify the metabolites whose concentrations change in the respiratory tree of sea cucumbers in response to multiple environmental challenges, including heat, hypoxia, and the combination of those two stressors. Balancing energy supplies and energy demands is the main challenge of survival for sea cucumbers in adverse environments. Thus, metabolic processes related to amino acids, carbohydrates, lipids, cofactors and vitamins, and nucleotides responded to the two types of stress, allowing the sea cucumber to maintain basic subsistence. Our present results revealed the significantly changed metabolites and key pathways associated with this response, which may be important for understanding how environmental changes affect the metabolism of the sea cucumber. Because of gaps in the literature, some mechanisms containing uncommon metabolites involved in responding to adverse environments need further study. In sea cucumbers, more metabolites responded to heat plus hypoxia than to either type of stress alone, illustrating the wide scope of the metabolic response that sea cucumbers would undergo when affected by global climate change. Our results provide insight into the responses of aquatic animals to global warming and oxygen deficiency.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2018.11.063.

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Conflicts of interest

The authors declare no conflict of interest.

References

Agriculture, M.o, 2016. China Fishery Statistical Yearbook. China Agriculture Press, Beijing.
Akram, M., 2014. Citric acid cycle and role of its intermediates in metabolism. Cell Biochem. Biophys. 68, 475–478.
Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., Tumlinson, J.H., 1997. An elicitor of plant volatiles from beet armyworm oral secretion. Science 276, 945–949.
An, Z., Dong, Y., Dong, S., 2007. Temperature effects on growth-ration relationships of juvenile sea cucumber *Apostichopus japonicus* (Selenka). Aquaculture 272, 644–648.
Bai, R., Paull, K.D., Herald, C.L., Malspeis, L., Pettit, G.R., Hamel, E., 1991. Halichondrin-B and homohalichondrin-B, marine natural-products binding in the vinca domain of tubulin - discovery of tubulin-based mechanism of action by analysis of differential cytotoxicity data. J. Biol. Chem. 266, 15882–15889.
Boardman, L., Sorensen, J.G., Kostal, V., Simek, P., Terblanche, J.S., 2016. Cold tolerance is unaffected by oxygen availability despite changes in anaerobic metabolism. Sci. Rep. 6.
Bowlus, R.D., Somero, G.N., 1979. Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. J. Exp. Zool. 208, 137–151.
Brose, S.A., Marquardt, A.L., Golovko, M.Y., 2014. Fatty acid biosynthesis from glutamate and glutamine is specifically induced in neuronal cells under hypoxia. J. Neurochem. 129, 400–412.
Chang, Y.Q., Ding, J., Song, J., 2004. The Biology Research and Cultivation of Sea Cucumber and Sea Urchins. Marine Publishing Company, pp. 1–4.
Cordero, C.P., Gomez-Gonzalez, S., Leon-Acosta, C.J., Morantes-Medina, S.J., Aristizabal, F.A., 2004. Cytotoxic activity of five compounds isolated from Colombian plants. Fitoterapia 75, 225–227.
Couch, J.F., 1936. Deltaline, a new alkaloid from *Delphinium occidentale* S. Wats. J. Am. Chem. Soc. 58, 684–685.
da Costa, R.C., Santana, D.B., Araujo, R.M., de Paula, J.E., do Nascimento, P.C., Lopes, N.P., Braz Filho, R., Espindola, L.S., 2014. Discovery of the rapanone and suberonone mixture as a motif for leishmanicidal and antifungal applications. Bioorg. Med. Chem. 22, 135–140.

de la Vega-Hernandez, K., Antuch, M., Cuesta-Rubio, O., Nunez-Figueredo, Y., Pardo-Andreu, G.L., 2017. Discerning the antioxidant mechanism of rapanone: a naturally occurring benzoquinone with iron complexing and radical scavenging activities. J. Inorg. Biochem. 170, 134–147.
Ellington, W.R., Hammen, C.S., 1977. Metabolic compensation to reduced oxygen tensions in the sea cucumber, *Sclerodactyla briareus* (Leseur). J. Comp. Physiol. 122, 347–358.
Ellis, R.P., Spicer, J.I., Byrne, J.J., Sommer, U., Viant, M.R., White, D.A., Widdicombe, S., 2014. 1H NMR metabolomics reveals contrasting response by male and female mussels exposed to reduced seawater pH, increased temperature, and a pathogen. Environ. Sci. Technol. 48, 7044–7052.
Evstratova, R.I., Rybalko, K.S., Razade, R.Y., 1967. Acroptilin — a new sesquiterpene lactone from *Acroptilon repens*. Chem. Nat. Compd. 3, 239.
Fasulo, S., Iacono, F., Cappello, T., Corsaro, C., Maisano, M., D'Agata, A., Giannetto, A., De Domenico, E., Parrino, V., Paro, G.L., 2012. Metabolomic investigation of *Mytilus galloprovincialis* (Lamarck 1819) caged in aquatic environments. Ecotoxicol. Environ. Saf. 84, 139–146.
Glaubitz, U., Erban, A., Kopka, J., Hincha, D.K., Zuther, E., 2015. High night temperature strongly impacts TCA cycle, amino acid and polyamine biosynthetic pathways in rice in a sensitivity-dependent manner. J. Exp. Bot. 66, 6385–6397.
Gonzalez, E., van Liempd, S., Conde-Vancells, J., Gutierrez-de Juan, V., Perez-Cormenzana, M., Mayo, R., Berisa, A., Alonso, C., Marquez, C.A., Barr, J., Lu, S.C., Mato, J.M., Falcon-Perez, J.M., 2012. Serum UPLC-MS/MS metabolic profiling in an experimental model for acute-liver injury reveals potential biomarkers for hepatotoxicity. Metabolomics 8, 997–1011.
Granstrom, T.B., Takata, G., Morimoto, K., Leisola, M., Izumori, K., 2005. L-Xylose and L-lyxose production from xylitol using Alcaligenes 701B strain and immobilized L-rhamnose isomerase enzyme. Enzym. Microb. Technol. 36, 976–981.
Green, B.T., Welch, K.D., Gardner, D.R., Stegelmeier, B.L., Pfister, J.A., Cook, D., Davis, T.Z., 2011. A toxicokinetic comparison of norditerpenoid alkaloids from *Delphinium barbeyi* and *D. glaucescens* in cattle. J. Appl. Toxicol. 31, 20–26.
Guo, L.P., Gu, Z.X., Jin, X.L., Yang, R.Q., 2017. iTRAQ-based proteomic and physiological analyses of broccoli sprouts in response to the stresses of heat, hypoxia and heat plus hypoxia. Plant Soil 414, 355–377.
Hallman, T.M., Rojas-Vargas, A.C., Jones, D.R., Richards, J.G., 2008. Differential recovery from exercise and hypoxia exposure measured using 31P-and 1H-NMR in white muscle of the common carp *Cyprinus carpio*. J. Exp. Biol. 211, 3237–3248.
Hanley, J.A., McNeil, B.J., 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143, 29–36.
Hines, A., Oladiran, G.S., Bignell, J.P., Stentiford, G.D., Viant, M.R., 2007. Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. Environ. Sci. Technol. 41, 3375–3381.
Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., 2007. Coral reefs under rapid climate change and ocean acidification. Science 318, 1737–1742.
Hu, T.M., deFreitas, A.S.W., Curtis, J.M., Oshima, Y., Walter, J.A., Wright, J.L.C., 1996. Isolation and structure of prorocentrolide B, a fast-acting toxin from *Prorocentrum maculosum*. J. Nat. Prod. 59, 1010–1014.
Huang, L., Nichols, L.A.B., Craig, J.K., Smith, M.D., 2012. Measuring welfare losses from hypoxia: the case of North Carolina brown shrimp. Mar. Resour. Econ. 27, 3–23.
Huo, D., Sun, L., Li, X., Ru, X., Liu, S., Zhang, L., Xing, L., Yang, H., 2017. Differential expression of miRNAs in the respiratory tree of the sea cucumber (*Apostichopus japonicus*) under hypoxia stress. G3: Genes|Genomes|Genetics 7, 3681–3692.
Huo, D., Sun, L., Ru, X., Zhang, L., Lin, C., Liu, S., Xin, X., Yang, H., 2018. Impact of hypoxia stress on the physiological responses of sea cucumber *Apostichopus japonicus*: respiration, digestion, immunity and oxidative damage. PeerJ 6, e4651.
Ji, T., Dong, Y., Dong, S., 2008. Growth and physiological responses in the sea cucumber, *Apostichopus japonicus* Selenka: aestivation and temperature. Aquaculture 283, 180–187.
Jones, O.A.H., Maguire, M.L., Griffin, J.L., Dias, D.A., Spurgeon, D.J., Svendsen, C., 2013. Metabolomics and its use in ecology. Austral Ecol. 38, 713–720.
Karakach, T.K., Huenupi, E.C., Soo, E.C., Walter, J.A., Afonso, L.O., 2009. 1H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress. Metabolomics 5, 123–137.
Keeling, R.F., Garcia, H.E., 2002. The change in oceanic O_2 inventory associated with recent global warming. In: Proceedings of the National Academy of Sciences. vol. 99. pp. 7848–7853.
Kolde, R., 2012. Pheatmap: pretty heatmaps. In: R Package Version 61.
Krajnc, D., Norton, H.N., Hadjiconstantinou, M., 1996. Glutamate, glutamine and glutamine synthetase in the neonatal rat brain following hypoxia. Brain Res. 707, 134–137.
Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., Kling, P., Koppe, W., Larsson, D.J., Björnsson, B.T., 2013. The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 164, 44–53.
Lardon, I., Eyckmans, M., Vu, T.N., Laukens, K., De Boeck, G., Dommisse, R., 2013. 1H-NMR study of the metabolome of a moderately hypoxia-tolerant fish, the common carp (*Cyprinus carpio*). Metabolomics 9, 1216–1227.
Le, A., Lane, A.N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C.J., Slusher, B.S., Zhang, H., 2012. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab. 15, 110–121.
Leite, J.S.M., Cruzat, V.F., Krause, M., Bittencourt, P.I.H.D., 2016. Physiological regulation of the heat shock response by glutamine: implications for chronic low-grade inflammatory diseases in age-related conditions. Nutrire 41, 17.
Li, G.R., Ren, L.H., Sun, G.H., Yang, J.M., Wei, X.M., Liu, Z.C., Li, S.J., Jiang, H., 2016. Effects of hypoxic stress on oxidative stress indices in *Apostichopus japonicus*. Prog. Fish. Sci. 37, 133–139.
Lin, C.Y., Viant, M.R., Tjeerdema, R.S., 2006. Metabolomics: methodologies and applications in the environmental sciences. J. Pestic. Sci. 31, 245–251.
Liu, Y., Li, F., Song, B., Sun, H., Zhang, X., Gu, B., 1996. Study on aestivating habit of sea cucumber *Apostichopus japonicus* Selenka: ecological characteristics of aestivation. J. Fish. Sci. China 3, 41–48.
Liu, G.S., Cai, X.Y., Tong, F., Wang, L., Zhang, X.M., 2014. Investigation of massive death of sea cucumber in artificial reef zone of Shuangdao Bay, Weihai. Fish. Inf. Strategy 29, 122–129.
Liu, S., Zhou, Y., Ru, X., Zhang, M., Cao, X., Yang, H., 2016. Differences in immune function and metabolites between aestivating and non-aestivating *Apostichopus japonicus*. Aquaculture 459, 36–42.
Lu, J., Shi, Y., Wang, S., Chen, H., Cai, S., Feng, J., 2016. NMR-based metabolomic analysis of *Haliotis diversicolor* exposed to thermal and hypoxic stresses. Sci. Total Environ. 545–546, 280–288.
Matear, R., Hirst, A., 2003. Long-term changes in dissolved oxygen concentrations in the ocean caused by protracted global warming. Glob. Biogeochem. Cycles 17.
Meng, Q., Zhang, T., Jiang, B., Mu, W.M., Miao, M., 2016. Advances in applications, metabolism, and biotechnological production of L-xylulose. Appl. Microbiol. Biotechnol. 100, 535–540.
Muniruzzaman, S., Pan, Y.T., Zeng, Y., Atkins, B., Izumori, K., Elbein, A.D., 1996. Inhibition of glycoprotein processing by L-fructose and L-xylulose. Glycobiology 6, 795–803.
Newman, D.J., 2016. Developing natural product drugs: supply problems and how they have been overcome. Pharmacol. Ther. 162, 1–9.
Newsholme, P., Procopio, J., Lima, M.M.R., Pithon-Curi, T.C., Curi, R., 2003. Glutamine and glutamate - their central role in cell metabolism and function. Cell Biochem. Funct. 21, 1–9.
Oka, H., Suzuki, S., Suzuki, H., Oda, T., 1976. Increased urinary-excretion of L-xylulose in patients with liver cirrhosis. Clin. Chim. Acta 67, 131–136.
Ospina, L.F., Calle, J., Arteaga, L., Pinzon, R., Alcaraz, M.J., Paya, M., 2001. Inhibition of acute and chronic inflammatory responses by the hydroxybenzoquinonic derivative rapanone. Planta Med. 67, 791–795.
Perez-Enciso, M., Tenenhaus, M., 2003. Prediction of clinical outcome with microarray data: a partial least squares discriminant analysis (PLS-DA) approach. Hum. Genet. 112, 581–592.
Portner, H.O., Langenbuch, M., Michaelidis, B., 2005. Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: from earth history to global change. J. Geophys. Res. Oceans 110.
Qian, Y., 2011. Experimental Studies on Different Dissolved Oxygen Levels and Exposure in Air Under Different Temperature in Sea Cucumber, *Apostichopus japonicus* Ocean University of China.
Riedel, B., Zuschin, Martin, Stachowitsch, Michael, 2012. Tolerance of benthic macrofauna to hypoxia and anoxia in shallow coastal seas: a realistic scenario. Mar. Ecol. Prog. Ser. 458 (39–+).
Ru, X., Zhang, L., Liu, S., Yang, H., 2017. Reproduction affects locomotor behaviour and muscle physiology in the sea cucumber, *Apostichopus japonicus*. Anim. Behav. 133, 223–228.
Sangster, T., Major, H., Plumb, R., Wilson, A.J., Wilson, I.D., 2006. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. Analyst 131, 1075–1078.
Sawada, Y., Yoshinaga, N., Fujisaki, K., Nishida, R., Kuwahara, Y., Mori, N., 2006. Absolute configuration of volicitin from the regurgitant of lepidopteran caterpillars and biological activity of volicitin-related compounds. Biosci. Biotechnol. Biochem. 70, 2185–2190.
Schock, T.B., Stancyk, D.A., Thibodeaux, L., Burnett, K.G., Burnett, L.E., Boroujerdi, A.F., Bearden, D.W., 2010. Metabolomic analysis of Atlantic blue crab, *Callinectes sapidus*, hemolymph following oxidative stress. Metabolomics 6, 250–262.
Shao, Y., Li, C., Ou, C., Zhang, P., Lu, Y., Su, X., Li, Y., Li, T., 2013. Divergent metabolic responses of *Apostichopus japonicus* suffered from skin ulceration syndrome and pathogen challenge. J. Agric. Food Chem. 61, 10766–10771.
Shao, Y., Li, C., Chen, X., Zhang, P., Li, Y., Li, T., Jiang, J., 2015. Metabolomic responses of sea cucumber *Apostichopus japonicus* to thermal stresses. Aquaculture 435, 390–397.
Shen, B.Z., Zheng, Z.W., Dooner, H.K., 2000. A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: characterization of wild-type and mutant alleles. Proc. Natl. Acad. Sci. USA 97, 14807–14812.
Sher, P.K., Hu, S., 1990. Increased glutamate uptake and glutamine synthetase activity in neuronal cell cultures surviving chronic hypoxia. Glia 3, 350–357.
Sicher, R., 2013. Combined effects of CO_2 enrichment and elevated growth temperatures on metabolites in soybean leaflets: evidence for dynamic changes of TCA cycle intermediates. Planta 238, 369–380.
Sondergaard, T.E., Hansen, F.T., Purup, S., Nielsen, A.K., Bonefeld-Jorgensen, E.C., Giese, H., Sorensen, J.L., 2011. Fusarin C acts like an estrogenic agonist and stimulates breast cancer cells in vitro. Toxicol. Lett. 205, 116–121.

Sui, X., Liao, Y., 1988. Culture and Enhance of Sea Cucumber. Agriculture Press, Beijing, China.
Sun, R.C., Denko, N.C., 2014. Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. Cell Metab. 19, 285–292.
Sun, L., Sun, J., Xu, Q., Li, X., Zhang, L., Yang, H., 2017. Metabolic responses to intestine regeneration in sea cucumbers *Apostichopus japonicus*. Comp. Biochem. Physiol. D Genomics Proteomics 22, 32–38.
Tomlins, R.I., Pierson, M.D., Ordal, Z.J., 1971. Effect of thermal injury on the TCA cycle enzymes of *Staphylococcus aureus* MF 31 and *Salmonella typhimurium* 7136. Can. J. Microbiol. 17, 759–765.
Tuffnail, W., Mills, G.A., Cary, P., Greenwood, R., 2009. An environmental 1H NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation. Metabolomics 5, 33–43.
Uemura, D., Takahashi, K., Yamamoto, T., Katayama, C., Tanaka, J., Okumura, Y., Hirata, Y., 1985. Norhalichondrin-a - an antitumor polyether macrolide from a marine sponge. J. Am. Chem. Soc. 107, 4796–4798.
Viant, M.R., Rosenblum, E.S., Tjeerdema, R.S., 2003. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. Environ. Sci. Technol. 37, 4982–4989.
Vuong, C., Kidder, J.B., Jacobson, E.R., Otto, M., Proctor, R.A., Somerville, G.A., 2005. Staphylococcus epidermidis polysaccharide intercellular adhesin production significantly increases during tricarboxylic acid cycle stress. J. Bacteriol. 187, 2967–2973.
Wei, L., Wang, Q., Ning, X., Mu, C., Wang, C., Cao, R., Wu, H., Cong, M., Li, F., Ji, C., 2015. Combined metabolome and proteome analysis of the mantle tissue from Pacific oyster *Crassostrea gigas* exposed to elevated pCO_2. Comp. Biochem. Physiol. D Genomics Proteomics 13, 16–23.
Wischmeyer, P.E., Vanden Hoek, T.L., Li, C., Shao, Z., Ren, H., Riehm, J., Becker, L.B., 2003. Glutamine preserves cardiomyocyte viability and enhances recovery of contractile function after ischemia-reperfusion injury. JPEN J. Parenter. Enteral Nutr. 27, 116–122.
Xiong, A., Yang, L., Ji, L., Wang, Z., Yang, X., Chen, Y., Wang, X., Wang, C., Wang, Z., 2012. UPLC-MS based metabolomics study on *Senecio scandens* and S. vulgaris: an approach for the differentiation of two Senecio herbs with similar morphology but different toxicity. Metabolomics 8, 614–623.
Xu, D., Sun, L., Liu, S., Zhang, L., Ru, X., Zhao, Y., Yang, H., 2014. Molecular cloning of heat shock protein 10 (Hsp10) and 60 (Hsp60) cDNAs and their expression analysis under thermal stress in the sea cucumber *Apostichopus japonicus*. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 171, 49–57.
Xu, D., Sun, L., Liu, S., Zhang, L., Yang, H., 2015. Histological, ultrastructural and heat shock protein 70 (HSP70) responses to heat stress in the sea cucumber *Apostichopus japonicus*. Fish Shellfish Immunol. 45, 321–326.
Xu, D., Zhou, S., Yang, H., 2017. Carbohydrate and amino acids metabolic response to heat stress in the intestine of the sea cucumber *Apostichopus japonicus*. Aquac. Res. 48, 5883–5891.
Yan, M.C., Shan, L.Z., Xie, Q.L., Lin, S.Z., Shao, X.B., Liu, W.C., Huang, X.K., 2008. Influence of temperature, salinity and body weight on oxygen consumption and ammonia excretion of *Oplegnathus fasciatus* juvenile. In: Advances in Marine Science.
Yang, H., Yuan, X., Zhou, Y., Mao, Y., Zhang, T., Liu, Y., 2005. Effects of body size and water temperature on food consumption and growth in the sea cucumber *Apostichopus japonicus* (Selenka) with special reference to aestivation. Aquac. Res. 36, 1085–1092.
Yang, H., Zhou, Y., Zhang, T., Yuan, X., Li, X., Liu, Y., Zhang, F., 2006. Metabolic characteristics of sea cucumber *Apostichopus japonicus* (Selenka) during aestivation. J. Exp. Mar. Biol. Ecol. 330, 505–510.
Yang, C., Ko, B., Hensley, C.T., Jiang, L., Wasti, A.T., Kim, J., Sudderth, J., Calvaruso, M.A., Lumata, L., Mitsche, M., 2014. Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. Mol. Cell 56, 414–424.
Yuan, X., 2005. Studies on Physio-ecology and Bioremediation of the Sea Cucumber, *Apostichopus japonicus* (Selenka) (Ph. D thesis). Graduate School of Chinese Academy of Science (138 pp., in Chinese with English abstract).
Zhang, L., Feng, Q., Ding, K., Sun, L., Huo, D., Fang, Y., Zhang, T., Yang, H., 2017. Genome-wide analysis of gene expression profile in the respiratory tree of sea cucumber (*Apostichopus japonicus*) in response to hypoxia conditions. J. Mar. Biol. Assoc. U. K. 1–10.
Zheng, H., Li, B., Rong, X., Liao, M., Chen, G., Zhang, Z., Wang, L., Wang, Y., Zhou, A., 2014. Effects of salinity and dissolved oxygen variation on the non-specific immune response of *Apostichopus japonicus*. Prog. Fish. Sci. 01, 118–124.
Zhou, B., Randall, D., Lam, P., Ip, Y., Chew, S., 2000. Metabolic adjustments in the common carp during prolonged hypoxia. J. Fish Biol. 57, 1160–1171.